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Cytotoxic and Tripanocide Activities of *Pityrogramma calomelanos* (L.) Link.

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ABSTRACT.—Chagas disease is caused by *Trypanosoma cruzi,* and is considered a public health problem. The current treatments for this disease are the synthetic drugs nifurtimox and benzonidazol, which are highly toxic. *Pityrogramma calomelanos,* a plant used in traditional medicine as an astringent, analgesic, anti-hemorrhagic, pectoral depurative, emmenagogue, anti-hypertensive, anti-pyretic and an anti-tussive was tested for antiepimastigote activity in vitro. An ethanol extract and hexane fraction of P. calomelanos was prepared and tested against *T. cruzi* (CL-B5 clone). The effective concentration capable of killing 50% of parasites (EC_{50}) was 55.26 µg/mL and 73.57 µg/mL for the ethanol extract and hexane fraction, respectively. This is the first record of tripanocidal activity for *P. calomelanos.* Our results indicate that *P. calomelanos* could be a source of antiepimastigote natural products with only moderate toxicity toward healthy human cells.

KEY WORDS.—Antiepimastigote activity, Chagas disease, cytotoxicity, *Pityrogramma calomelanos*

Developing countries with abundant traditional knowledge and rich biodiversity, as in the case of Brazil, still grapple with a high incidence of so-called "neglected diseases," such as tuberculosis, malaria and Chagas disease (Croft *et al.*, 2005); diseases that have the potential to be treated with natural products of plant origin from Brazil (Croft *et al.*, 2005). Brazil has the greatest biodiversity in the world, with more than 55,000 species of plants cataloged, and an estimated total of 550,000 species (Croft and Sundar, 2006), but only 8% have been studied in the search for bioactive compounds (Simões *et al.*, 2007).

Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi,* transmitted mainly by the insects from the genus *Triatoma,* representing a

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public health problem in South America with 20 million people infected and 90 million at risk in endemic areas (WHO, 2000). The parasite can be transmitted to humans by triatomine insects, foods contaminated with feces, blood, organ transplants, and by the transplacental route (Prata, 2001).

The treatment of this disease remains ineffective. Several compounds have been tested to evaluate their ability to eliminate infection by *T. cruzi* (Lana and Tafuri, 2005). Two drugs are currently used: nifurtimox and benzonidazole. These drugs are active against the parasite blood and tissue forms. However, side effects of these drugs are the most significant argument against their general use: the use of nifurtimox is associated with weight loss, psychic alterations and gastric problems; the use of benzonidazole is associated with cutaneous problems such as hypersensitivity, dermatitis, edema, fever, lymphadenopathy and muscle pain (Castro *et al.*, 2006).

Due to these side effects, there is an urgent need for the development of new drugs, and a source for these new drugs can be natural products with anti-*T. cruzi* activity (WHO, 2002). Coura and Castro (2002) reported that several herbal products, such as alkaloids, taxoids, estilbenoids, hormones, propolis, naphthoquinones and crude extracts from plants, have activity against *T. cruzi*. In their work, Menezes *et al.* (2005) related that coumarinic compounds with the highest binding affinity, such as chapelin, isolated from *Rutaceae* species promoted the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In this binding study, the affinity of the compounds was determined by their ability to displace NAD+ from the enzyme receptor site. The inhibition of this enzyme affects the process of energy generation by glycolysis in the amastigote forms of *T. cruzi*.

Pityrogramma calomelanos (L.) Link. (Pteridaceae), known in Brazil as "avenca-branca" or "avenca-preta" is used as an ornamental and medicinal plant. This fern has several biological properties reported in the literature such as astringent, analgesic, anti-hemorrhagic, anti-hypertensive, anti-pyretic, anti-tussive (Barros and Andrade, 1997). Many compounds from the leaves of *P. calomelanos* that have been isolated are flavonoids, [(8-phenylpropionyl)-5,7-dihydroxydihydroneoflavone] and {8-[3-(4-*p*-methoxyphenyl)porpionyl]-7-dihydrodihydroxyneoflavone} named calomelanol (Fujio *et al.*, 1991), terpens, as calomelanolactone (Victor *et al.*, 1978) and chalcones, as 2′,6′-dihydroxy-4′,4′-dihydroxychalcone (Sukuruman and Ramadasan, 1991). Due to the social and economic importance of Chagas disease and the absence of studies reporting on the effect of this fern against *T. cruzi*, the purpose of this work was to demonstrate the anti-*Trypanosoma* activity of Pityrogramma calomelanos.

Materials and Methods

Plant Material

Leaves of *Pityrogramma calomelanos* were collected in the rainy season (September, 2009) in the city of Crato, Ceará State, Brazil. The plant material

was identified by Dr. Antonio Álamo Feitosa Saraiva and the voucher specimen was deposited with the identification number 5570 in the Herbarium "Dárdano de Andrade Lima" of University of the Region of Cariri, Crato, CE, Brazil.

Preparation of Ethanol Extract (EEPC) and Hexane Fraction (HFPC) of Pityrogramma Calomelanos

Nine hundred fifty grams of leaves were dried and kept at room temperature. The powdered leaf material was macerated using 1 L of 95% ethanol as the solvent for 72 h at room temperature. The mixture was filtered and concentrated under vacuum in a rotary evaporator under 60°C and 760mm/Hg of temperature and pressure, respectively (Brasileiro *et al.*, 2006). Nine hundred fifty grams of aerial parts yielded 50 g of ethanol extract (EEPC). After this, 40 g of EEPC were dissolved in 95% ethanol mixed with silica gel (Merck®) and fractionated with hexane by percolation with the solvent. The hexane fraction (HFPC) was concentrated under vacuum in a rotary evaporator under 60°C and 760 mm/Hg of temperature and pressure, producing 0.56 g of HFPC. The hexane was chosen to extract nonpolar compounds of the EEPC. After this, the ethanol extract and hexane fraction were diluted using 1 mL of DMSO to the assays.

Phytochemical Screening

The qualitative phytochemical assays were performed to detect the presence of secondary metabolites. The tests are based in the visual observation of color modifications or by precipitation after the use of reagents to detect each group of secondary metabolites. The tests were performed according Matos (2009).

Quantification of Phenolics Compounds of Hexane Fraction by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively, following the method described by Laghari *et al.* (2011), with slight modifications. The fern extract was analyzed and dissolved in hexane at a concentration of 3 mg/mL. The presence of six phenolic compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume 40 μL and the wavelengths were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The hexane fraction and mobile phase were filtered through 0.45 μm

membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in the HPLC mobile phase at a concentration range of 0.020–0.200 mg/mL for kaempferol, quercetin and rutin; and 0.050–0.250 mg/mL for gallic, caffeic and chlorogenic acids. The chromatography peaks were confirmed by comparing retention time with those of reference standards and by DAD spectra (200–400 nm). All chromatography operations were carried out at ambient temperature and in triplicate.

Cell Strains Used

For *in vitro* studies of *Trypanosoma cruzi*, the clone CL-B5 was used (Buckner *et al.*, 1996). The stable parasite, transfected with *Escherichia coli* (*lacZ*) that have the gene coding for β-galactosidase, was kindly provided by Dr. F. Buckner through Instituto Conmemorativo Gorgas (Panama). Epimastigotes were grown at 28°C in liver infusion tryptose broth (Difco, Detroit, MI) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (Reig Jofr'e S.A., Barcelona, Spain), as described previously (Le Senne *et al.*, 2002), and harvested during the exponential growth phase.

Murine J774 macrophages were grown in plastic 25 μL flasks in RPMI 1640 medium (Sigma®; with glutamine, without bicarbonate and phenol red indicator), supplemented with 20% heat inactivated (30 min, 56°C) fetal bovine serum (FBS) and penicillin G (100 U/mL) and streptomycin (100 μg/mL) in a humidified 5% CO_2/95% air atmosphere at 37° C. For the experiments, cells in the pre-confluence phase were harvested with trypsin. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Cell viability was evaluated colorimetrically with resazurin according to a previously described method (Rolon *et al.*, 2006).

Reagents

Chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in 0.9% Triton X- 100 (pH 7.4). Penicillin G (Ern, S.A., Barcelona, Spain), streptomycin (Reig Jofre S.A., Barcelona, Spain). Resazurin sodium salt was obtained from Sigma-Aldrich (St Louis, MO) and stored at 4°C protected from light. A solution of resazurin was prepared in 1% phosphatebuffered solution (PBS), pH 7, and filter sterilized prior to use.

Epimastigote Susceptibility Assay

The screening assay was performed in 96-well microplates with cultures that had not reached the stationary phase, as described (Vega *et al.*, 2005). Briefly, epimastigotes were seeded at 1×10^5 mL^{-1} in 200 μL of liver tryptose broth medium. The plates were then incubated with EEPC and HFPC (0.1–50 μg/mL) at 28°C for 72 h, at which time 50 μL of CPRG solution was added to give a final concentration of 200 μM. the plates were incubated at 37°C for an additional 6 h and were then read at 595 nm. Nifurtimox was used as the reference drug. Each

concentration was tested in triplicate. Each experiment was performed twice separately. The efficacy of each compound was estimated by calculating the antiepimatigote percent (AE%).

Cytotoxicity Assays

Murine J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottom microplates with 100 µL of RPMI 1640 medium. The cells were allowed to attach for 24 h at 37°C, 5% CO_2, after which the medium was removed and replaced with medium containing different concentrations of treatment. Macrophages were incubated with treatment for another 24 h. Growth controls were also included. Afterwards, 20 µL of 2 mM resazurin solution was added and plates were returned to incubator for another 3 h. to evaluate cell viability. The reduction of resazurin was determined by dual wavelength absorbance measurement at 490 nm and 595 nm. Background was subtracted. Each concentration was assayed three times. Blanks include medium and treatment only. The cytotoxicity of each compound was estimated by calculating the cytotoxic percentage (EC_{50}), cells surviving/cells dead.

Statistical Analysis

The statistical analysis was performed using Prism program 5.0. The effective concentration (EC_{50}) was calculated by the linear regression method.

Results and Discussion

The phytocompounds are shown in Table 1. Two groups of substances were observed in the hexane fraction but not detected in the ethanol extract: flavonols and chalcones.

HPLC Analysis

Due to the results obtained with the hexane fraction, this product was subjected to HPLC analysis for quantification of phenol compounds. HPLC fingerprinting of hexane fraction of *Pityrogramma calomelanos* revealed the presence of the gallic acid (t_R = 17.83 min; peak 1), chlorogenic acid (t_R = 28.14 min; peak 2), caffeic acid (t_R = 34.09 min; peak 3), quercetin (t_R = 49.78 min; peak 5) and kaempferol (t_R = 58.96 min; peak 6) (Fig. 1 and Table 2). The HPLC analysis, according to the chemical analysis, revealed the presence of flavonoids (quercetin and kaempferol) and phenolic acids (chlorogenic and caffeic acids) in the HFPC.

Our results indicate that HFPC, when tested at a concentration of 50 µg/mL, was active against epimastigote forms of *T. cruzi*, killing 71% of the parasites with 0% cytotoxicity. This article is the first report relating the chemical composition of *P. calomelanos* with the trypanocidal activity. Hayacibara *et al.* (2005) reported that the chemical composition of propolis (a resinous mixture collected by honey bees from tree buds, flowers or other botanical sources) is

TABLE 1. Phytochemical screening of ethanol extract and hexane fraction of *Pityrogramma calomelanos* (L.) Link.

	METABOLITES														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EEPC	+	−	−	+	+	+	+	+	−	−	+	−	−	−	+
HFPC	−	−	−	−	+	−	+	+	+	−	+	−	+	−	−

1 – Alkaloids; 2 – Anthocyanidins; 3 – Anthocyanins; 4 – Aurones; 5 – Catechins; 6 – Chalcones; 7 – Flavones; 8 – Flavonones; 9 – Flavonols; 10 – Flavononols; 11 – Phenols; 12 – Leucoanthocyanidins; 13 Xanthones – 14 – Pyrogallics Tannins; 15 – Saponins; (+) presence; (−) absence; EEPC (ethanol extract of *Pityrogramma calomelanos*); HFPC (hexane fraction of *Pityrogramma calomelanos*).

rich in phenylpropanoids (chlorogenic and caffeic acids) and Prytzyk *et al.* (2003), tested the *in vitro* activity of the propolis extract against *Trypanosoma cruzi*, detecting an interesting activity against epimastigote forms of this parasite. These results are similar to our work, showing a relationship between phenylpropanoids and tripanocidal activity. Chalcones isolated from *Myrcia hiemales* Camb. demonstrated an inhibitory effect against the cruzaine (Deise *et al.*, 2006; Silva, 2007). This is one of the most important cysteine-proteases in *Trypanosoma cruzi*, being essential for the parasite multiplication (Simeonov, 2008).

Several biological activities associated with flavonoids have been reported. Quercetin, the most common flavonoid detected in foods, presented antiprotozoal effects against *Plasmodium falciparum, Leishmania donovani, Trypanosoma brucei* and *T. rhodesiense* (Camacho *et al.*, 2002; Williamson and Finnigan,

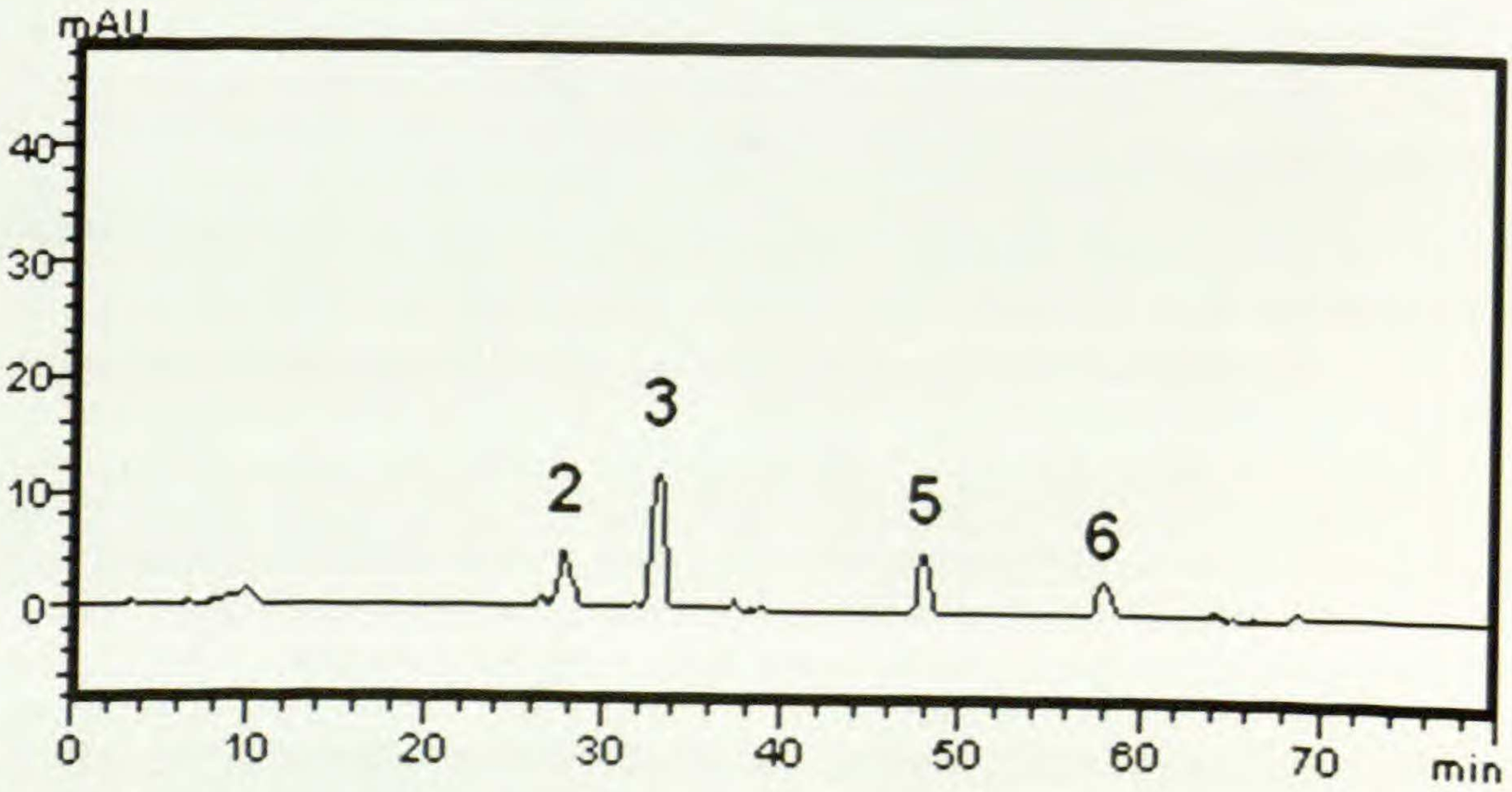

FIG. 1. Representative high performance liquid chromatography profile of (HFPC), detection UV was at 327nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6).

TABLE 2. Phenolics and flavonoids composition of hexane fraction of *Pityrogramma calomelanos* (L.) Link.

	HFPC	
Compounds	mg/g	Percent
Gallic acid	-	-
Chlorogenic acid	1.05 ± 0.01 a	0.10
Caffeic acid	4.59 ± 0.03 b	0.45
Rutin	-	-
Quercetin	1.14 ± 0.02 a	0.11
Kaempferol	0.93 ± 0.01 a	0.09

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.005$.

1978). Ana *et al.* (2010), demonstrated that quercetin and taxifolin, isolated from a hexane fraction of *Rapanea lancifolia* (Mart.) Mez, demonstrated a low trypanocidal activity. Takeara *et al.* (2003) evaluated the quercetin-3-methyl ether isolated of extract from *Lychnophora staavioides* Mart. (Asteraceae), demonstrating a promising potential for the use against *T. cruzi*, not affecting the blood cells.

Trypanosoma cruzi Epimastigote Susceptibility Assay

The trypanocidal activity of EEPC and HFPC is shown in Table 3. The results demonstrated that HFPC was active against the strain CL-B5 strain of *T. cruzi* with a concentration 50 μg/mL, inhibiting 71% for *T. cruzi* (EC_{50} = 73.57μg/mL). This is impressive due the fact that an EC_{50} lower than 500 μg/mL is considered clinically relevant (Rosas *et al.*, 2007). However, the ethanol extract of *P. calomelanos* did not show a clinically relevant inhibitory activity against the epimastigote forms due its toxicity against murine macrophages.

Other plants of the Brazilian flora have shown trypanocidal activity, such as extracts and fractions of *Ampelozizyphus amazonicus* Ducke (Rosas *et al.*, 2007), the ethyl acetate fraction of *Camellia sinensis* (L.) Kuntze (Paveto *et al.*, 2004) and polar extracts of *Siphoneugena densiflora* O. Berg (Gallo *et al.*, 2008).

Cytotoxic Activity

Also important in the search for active compounds with trypanocidal activity is the toxicity against mammalian host cells. J774 macrophages were utilized to evaluate the cytotoxity and determine the selectivity of EEPC and HFPC. The results are presented in Table 2. No toxicity was observed at concentrations of 5, 12, 25, and 50 μg/mL of the hexane fraction. The HFPC showed a low toxicity against macrophages J774 when compared with Roldos *et al.* (2008). In this study, the semi-synthetic compound 1,4-Hydroxylunularin, a derivative hydroxybibenzyl showed a toxic effect against 18.88% of

TABLE 3. Percent of parasite lysis induced by ethanol extract and hexane fraction of *Pityrogramma calomelanos* against the epimastigote forms of *Trypanosoma cruzi* CL-B5 strain.

Extract	Concentration (µg/mL)	%AE	%SD	% C	EC_{50}
EEPC	500	91	3.3	100	55.26
	200	77	3.1	86	
	100	74	2.7	73	
	50	37	1.8	6	
HFPC	50	71	1.2	0	73.57
	25	67	0.7	0	
	12.5	47	0.9	0	
Nifurtimox	10	89.1	3.3	-	0.91
	1	54.9	0.7	-	
	0.5	45.6	4.2	-	

%AE – Percent of inhibition of epimastigote forms; %SD – Standard deviation; % C – cytotoxic effect; EC_{50} – Concentration with 50% of the maximum activity; EEPC (ethanol extract of *P. calomelanos*), HFPC (hexane fraction of *P. calomelanos*).

macrophages in a concentration of 21.7 µg/mL. The hexane fraction (HFPC) of *P. calomelanos* appears to be promising in the development of new drugs to treat *T. cruzi*, mainly due to the low toxic effect *in vitro* and due the antiepimastigote activity demonstrated in our work, indicating the necessity to proceed with *in vivo* studies.

Conclusion

Our results indicate that *Pityrogramma calomelanos* could be a source of plant-derived natural products with antiepimastigote activity and low toxicity, representing an interesting alternative to other efforts to combat infectious diseases such Chagas disease.

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